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The Absence of Pupylation (Prokaryotic Ubiquitin-Like Protein Modification) Affects Morphological and Physiological Differentiation in *Streptomyces coelicolor*

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ABSTRACT

Protein turnover is essential in all living organisms for the maintenance of normal cell physiology. In eukaryotes, most cellular protein turnover involves the ubiquitin-proteasome pathway, in which proteins tagged with ubiquitin are targeted to the proteasome for degradation. In contrast, most bacteria lack a proteasome but harbor proteases for protein turnover. However, some actinobacteria, such as mycobacteria, possess a proteasome in addition to these proteases. A prokaryotic ubiquitination-like tagging process in mycobacteria was described and was named pupylation: proteins are tagged with Pup (prokaryotic ubiquitin-like protein) and directed to the proteasome for degradation. We report pupylation in another actinobacterium, *Streptomyces coelicolor*. Both the morphology and life cycle of *Streptomyces* species are complex (formation of a substrate and aerial mycelium followed by sporulation), and these bacteria are prolific producers of secondary metabolites with important medicinal and agricultural applications. The genes encoding the pupylation system in *S. coelicolor* are expressed at various stages of development. We demonstrated that pupylation targets numerous proteins and identified 20 of them. Furthermore, we established that abolition of pupylation has substantial effects on morphological and metabolic differentiation and on resistance to oxidative stress. In contrast, in most cases, a proteasome-deficient mutant showed only modest perturbations under the same conditions. Thus, the phenotype of the *pup* mutant does not appear to be due solely to defective proteasomal degradation. Presumably, pupylation has roles in addition to directing proteins to the proteasome.

IMPORTANCE

***Streptomyces* spp. are filamentous and sporulating actinobacteria, remarkable for their morphological and metabolic differentiation. They produce numerous bioactive compounds, including antifungal, antibiotic, and antitumor compounds. There is therefore considerable interest in understanding the mechanisms by which *Streptomyces* species regulate their complex physiology and production of bioactive compounds. We studied the role in *Streptomyces* of pupylation, a posttranslational modification that tags proteins that are then directed to the proteasome for degradation. We demonstrated that the absence of pupylation had large effects on morphological differentiation, antibiotic production, and resistance to oxidative stress in *S. coelicolor*. The phenotypes of pupylation and proteasome-defective mutants differed and suggest that pupylation acts in a proteasome-independent manner in addition to its role in proteasomal degradation.**

Turnover of cellular proteins is required in all living organisms to maintain normal cellular physiology. In eukaryotes, the ubiquitin-proteasome pathway is the major route of protein degradation and turnover (1). Ubiquitin is a 76-residue protein that can be conjugated to target proteins, marking them for degradation by the 26S proteasome complex. In addition to its role in tagging proteins for degradation, ubiquitination is also involved in protein transport and localization. It thereby contributes to regulating various cellular functions such as transcription, stress responses, apoptosis, and DNA repair (1, 2).

In bacteria, proteolysis is due to several proteases possessing an AAA^+ (ATPases associated with a variety of cellular activities) domain, including ClpAP, ClpXP, Lon, HslUV, and FtsH (3, 4). A bacterial 20S proteasome has also been described for some bacteria, including actinobacteria such as *Rhodococcus erythropolis* (5), *Streptomyces coelicolor* (6), *Frankia alni* (7), *Mycobacterium smegmatis* (8), and *Mycobacterium tuberculosis* (9). Unlike its eukaryotic counterpart, the actinobacterial proteasome is not essential (8–11), although its inactivation can be associated with various

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deleterious effects. For example, proteasome inactivation in *M. tuberculosis* impairs growth on agar media and reduces virulence in mice (9, 10). Conversely, proteasome mutants of *Streptomyces lividans* and *M. smegmatis* were described to be phenotypically indistinguishable from the wild-type strains under both normal and stress-inducing conditions (8, 11). Proteasome inactivation in *S. coelicolor* has only a modest effect on cell physiology: it was reported to increase resistance to cumene hydroperoxide, an effect associated with increased levels of a haloperoxidase enzyme (12). In addition, recent results suggest that the proteasome in *S. coelicolor* has a role in cell differentiation: proteasome-deficient mutants produce smaller amounts of pigmented antibiotics than the wild type and are delayed in their morphological development (13).

The roles of these bacterial proteasomes were unclear until a protein-tagging system specifically directing proteins for proteasomal degradation was discovered in mycobacteria (14, 15). This ATP-dependent pathway shares many functional similarities with the eukaryotic ubiquitin pathway (16). However, the sequence and structure of the modifier peptide, named Pup (prokaryotic ubiquitin-like protein), as well as the conjugation process differ from those of the ubiquitin pathway (16–20). In *M. tuberculosis* and *M. smegmatis*, Pup possesses a conserved C-terminal GGQ motif that requires deamidation of glutamine to glutamate by Dop (deamidase of Pup). PafA (proteasome accessory factor A) catalyzes isopeptide bond formation between the C-terminal glutamate of Pup and the ε-amino group of a lysine in the target protein (4).The pupylated protein is recognized and unfolded by Mpa (*Mycobacterium* proteasomal ATPase) and then degraded in the proteasome (21–23). Dop is also able to remove Pup from pupylated proteins, a process named depupylation (18, 24, 25).

Following the discovery of pupylation, several proteome-wide studies reported the identity of Pup-tagged proteins (pupylome) in mycobacteria (26–28). Pupylation was also demonstrated in *Rhodococcus erythropolis* (29) and in *Corynebacterium glutamicum* (30), and proteins targeted by pupylation in these organisms have been identified. Pupylated targets are numerous and belong to various functional classes of proteins. The mycobacterial genes *pafA* and *pup* (gene modified to encode a protein with a GGE C terminus) have been expressed in *Escherichia coli*, resulting in the pupylation of host proteins (31). This provides clear evidence that no mycobacterial cofactor is essential for substrate recognition by PafA.

To date, pupylation has been studied mainly in mycobacteria. Nevertheless, several actinobacteria, including streptomycetes, carry orthologues of *pup*, *dop*, and *pafA* that are likely to be involved in pupylation (14, 32). *Streptomyces* species are filamentous bacteria that undergo complex morphological differentiation and produce a great variety of secondary metabolites, including antibiotics, with important applications in human medicine and in agriculture. Both secondary metabolism and morphological differentiation are controlled by an intricate regulatory network; this network has been extensively studied in the reference strain of *Streptomyces coelicolor* (33, 34). Proteolysis is one of the mechanisms by which the differentiation of *Streptomyces* is regulated. For example, deficiency in the ATP-dependent Clp protease in *S. lividans* alters morphological differentiation (35), and BldD and SigT, key regulators of *Streptomyces* development, are subject to specific proteolytic control (13, 36). These various observations led us to study pupylation in *S. coelicolor*.

Here, we present an investigation of pupylation in *S. coelicolor*, including analyses of the transcription of genes involved in pupylation and the identification of some pupylated substrates. We also studied the effects of *pup* deletion particularly on morphological differentiation, secondary metabolism, and resistance to oxidative stress. We demonstrate that pupylation plays an important role in both sporulation and antibiotic production, revealing an additional level of developmental regulation in *S. coelicolor*. The phenotypes of *pup* and proteasome mutants suggest that pupylation is part of normal cell physiology through both proteasome-dependent and -independent actions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Table S1 in the supplemental material lists strains and plasmids used in this work. *S. coelicolor* A3(2) strain M145 (wild type) and mutant strains were grown on standard media and manipulated according to well-established protocols (37). *Escherichia coli* strains were grown in LB medium. *E. coli* DH5α was used as a host for plasmid constructions according to standard procedures (38). *E. coli* ET12567/pUZ8002 was used for conjugation of shuttle vectors from *E. coli* to *S. coelicolor* (37).

Construction of a *S. coelicolor* proteasome mutant by *prcB* disruption. Table S2 in the supplemental material lists the oligonucleotides used in this work. A proteasome-deficient strain was constructed by disrupting the *prcB* gene. An internal fragment of *prcB* (557 nucleotides [nt] of the 846-nt *prcB* coding sequence) was amplified (with primers HBP88 and HBP89) and ligated into the pGEM-T Easy vector. The EcoRI-*prcB*-EcoRI fragment was obtained by digestion with EcoRI and inserted into the single EcoRI site of pOJ260, yielding pOJ260-*prcB*. This construct was transferred from *E. coli* to *S. coelicolor* by conjugation. The *S. coelicolor prcB* disruption mutant was selected for apramycin resistance and analyzed by appropriate PCR amplifications, with verification by sequencing. We showed, by reverse transcription-PCR (RT-PCR), that the insertion into *prcB* abolished *prcA* transcription (data not shown), as expected, if the two genes were cotranscribed. We checked the stability of this mutant by culturing it for 5 days without selective pressure. We tested 500 clones from this culture, all of which were found to be resistant to apramycin.

Construction of a *S. coelicolor pup* deletion mutant. Upstream and downstream regions of *pup* were amplified from *S. coelicolor* genomic DNA by PCR and inserted into pGEM-T Easy (yielding pGEM-T-Easy-*pup*BG and pGEM-T-Easy-*pup*BD). We checked for an absence of mutations in the up- and downstream fragments by sequencing. The upstream and downstream fragments were inserted, together with the apramycin resistance cassette *att3-aac* (39), into the suicide vector pOSV400 (carrying a hygromycin resistance gene), yielding pOSV400-*pup*. In this plasmid, the *pup* upstream and downstream fragments flank the apramycin resistance gene. The pOSV400-*pup* plasmid was introduced into *S. coelicolor* by conjugation. *S. coelicolor* transconjugants resistant to apramycin were selected and screened for hygromycin sensitivity. The replacement of the *pup* gene with the apramycin resistance cassette was checked by PCR amplification (primers HBP5/L-acc2R and R-acc2F/HBP6). The apramycin resistance cassette was then excised by site-specific recombination, as previously described (39). The resulting *pup* locus was then amplified by PCR and sequenced (with the HBP5/HBP6 primers) to confirm that 206 bp had been deleted from the *pup* coding sequence and replaced with a 41-bp sequence.

Construct for the expression of His_6-Pup. The coding sequence of *pup* was amplified from *S. coelicolor* genomic DNA (see Table S2 in the supplemental material for primers). The pSET-E*-His-*pup* construct was obtained by amplification with primers NS9 and NS12, generating an NdeI-His-*pup*-BamHI fragment. Primer NS9 was used to introduce a sequence encoding a hexahistidine tag upstream from the modified *pup* start codon. The NdeI-His-*pup*-BamHI fragment was inserted between the NdeI/BamHI sites in pHM11a such that the *pup* gene was under the control of the *ermE** promoter. The *ermE**-His-*pup* fragment was excised and inserted into the integrative vector pSET152 (XbaI/BamHI sites); the

resulting plasmid was introduced into *S. coelicolor* wild-type or Δ*pup* strains by conjugation (37).

SDS-PAGE and immunoblot analysis. Protein samples were prepared from 12 ml of cultures grown at 30°C in yeast extract-malt extract (YEME) or R2YE (37) liquid medium for 45 h. The mycelium was ground with glass beads (size, <106 μm; Sigma-Aldrich) in a FastPrep shaker (40 s at a 4.5 intensity; Q-BIOgene), and the extracts were clarified by centrifugation (10 min at 12,000 rpm). Protein concentrations in supernatants were determined by using the Bio-Rad protein assay, and samples were treated with Laemmli buffer and XT reducing agent (Bio-Rad).

SDS-PAGE was performed by using Criterion XT Bis-Tris precast 12% gels (Bio-Rad) with a Criterion electrophoresis system (Bio-Rad). Molecular size reference markers were obtained from Bio-Rad.

For immunoblot experiments, proteins were transferred from the gel to Hybond ECL nitrocellulose membranes by using a semidry transfer system (Bio-Rad). Immunodetection was based on Penta-His horseradish peroxidase (HRP) conjugate antibodies (Qiagen) and the Pierce ECL Western blotting substrate (Thermo Scientific), according to the manufacturers' protocols.

Preparation of pupylated targets. Strains were grown in 500 ml YEME (5 mM $MgCl_2$) or R2YE liquid medium for 45 h in baffled Erlenmeyer flasks. The mycelium was harvested by centrifugation and washed once with 50 ml of buffer I (50 mM $NaPO_4$, 300 mM NaCl, 20 mM imidazole [pH 8]). The mycelium was then suspended in buffer I (25 ml) and disrupted with a French press. Cell debris was removed by centrifugation, and the supernatant was filtered through 0.2-μm-pore-size membranes (Millipore). Crude extracts were then loaded onto a 0.3-ml Ni-nitrilotriacetic acid (NTA)-agarose column (Qiagen) previously equilibrated with buffer I, and the column was washed with buffer II (50 mM $NaPO_4$, 300 mM NaCl, 30 mM imidazole [pH 6.5]). The proteins were eluted with an imidazole gradient (30 to 500 mM) and recovered in 300-μl fractions. Elution was monitored by using a Uvicord SII UV detector (Pharmacia), and fractions were analyzed by SDS-PAGE and Coomassie blue staining. Selected fractions were pooled in a Slide-A-Lyzer cassette with a 3,500-molecular-weight cutoff (MWCO) (Thermo Scientific), dialyzed twice with 1 liter of ammonium acetate (300 mM, pH 7), and then dried in a Speed-Vac concentrator.

Mass spectrometry analysis, database searches, and protein identification. Dried protein fractions were reconstituted in a solution containing 100 mM Tris HCl (pH 8.5) and 8 M urea, reduced [5 mM Tris(2-carboxyethyl)phosphine], and alkylated (10 mM iodoacetamide). Endoproteinase Lys-C (Promega, Madison, WI, USA) was added at a 1:40 (wt/wt) dilution. The sample was incubated for 5 h at 37°C and then diluted 1:4 with 100 mM Tris HCl (pH 8.5). Trypsin Gold (mass spectrometry [MS] grade; Promega, Madison, WI, USA) was added at a 1:100 (wt/wt) dilution, and the digestion was allowed to proceed overnight at 37°C with shaking. The same quantity of enzyme was then added, and the sample was incubated for 5 h at 37°C with shaking. The sample was desalted with Omix C_{18} solid-phase extraction pipette tips (Varian), dried, and reconstituted in H_2O-acetonitrile-formic acid (98:2:0.1).

The resulting trypsin-digested peptides were analyzed by nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 system (Dionex, Amsterdam, Netherlands) coupled to an LTQ-Orbitrap Velos instrument. Aliquots of 5 μl of each sample were loaded onto a C_{18} precolumn (300-μm inner diameter by 5 mm; Dionex) at 30 μl/min in a solution containing 2% ACN and 0.1% FA. After 5 min of desalting, the precolumn was switched online with a 3-μm analytical PepMap100 C_{18} column (Dionex) equilibrated in 95% solvent A (2% ACN, 0.1% FA) and 5% solvent B (80% ACN, 0.08% FA). The peptides were eluted with a 2 to 60% gradient of solvent B over 37 min at a flow rate of 300 nl/min. The LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific, Bremen, Germany) was operated in the data-dependent acquisition mode with XCalibur software (Thermo Fisher Scientific, Bremen, Germany). Survey scan mass spectra were acquired with the Orbitrap instrument in the range of 300 to 2,000 *m/z* with the resolution set to a value of 60,000 at *m/z* 400. The 20 most intense ions per survey scan were selected for collision-induced dissociation (CID) fragmentation, and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was employed within 20 s and repeated after 30s to prevent repetitive selection of the same peptide.

The Mascot v.2.4.1 search engine was used with Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific, Bremen, Germany) to search a database of concatenated *S. coelicolor* proteins (Swiss-Prot) and known contaminants and reversed sequences of all entries (8,286 entries in total). The following search parameters were applied: carbamidomethylation of cysteines was set as a fixed modification, and pupylation of lysine (+243.086 Da due to the addition of a deamidated QGG motif [15]), oxidation of methionine, and carbamylation of lysine were set as variable modifications. The specificity of trypsin digestion was set for cleavage after Lys or Arg, and two missed cleavage sites were allowed. The mass tolerances for MS and MS/MS were set to 10 ppm and 0.5 Da, respectively. Scaffold software (version Scaffold_3.5.1; Proteome Software Inc., Portland, OR) was used for probability assignment and validation.

The raw data files were converted into mgf format by using Proteome Discoverer and analyzed with the InSpecT open-source search engine (http://proteomics2.ucsd.edu/LiveSearch/index.jsp), which was developed at the UCSD NIH NCRR center for computational mass spectrometry. The searches were performed with the same information for enzymes and modifications.

Scanning electron microscopy (SEM). *Streptomyces* strains were cultured for 5 days on a cellophane membrane placed on top of solid agar medium. Strips (4 cm by 2 cm) of the cellophane with well-developed mycelium growth were cut out and mounted onto glass slides, attached by Scotch tape. The slides were placed into a desiccator with a small container of 2% OsO_4 in double-distilled water (ddH_2O) and left for the mycelia to be fixed in osmium vapor for several days. Fixed and air-dried samples were sputter coated with gold using a Polaron SC510 sputter coater (Quorum Technologies Ltd., Lewes, United Kingdom) and examined with an Aquasem scanning electron microscope (Tescan, Brno, Czech Republic) in high-vacuum mode. The images of mycelia were randomly taken at both the periphery and central parts of the mycelium. Selected samples were reexamined at higher resolution with a Tescan Vega LSU scanning electron microscope (Tescan, Brno, Czech Republic) at 20 kV.

Determination of secondary metabolite production. *S. coelicolor* produces several secondary metabolites, including blue-pigmented actinorhodin (ACT), red-pigmented prodiginines (RED), and nonpigmented calcium-dependent antibiotic (CDA) (37). ACT and RED were assayed in R2YE liquid medium (40). About 10^{10} spores were used to inoculate 50 ml of R2YE medium in a 250-ml baffled flask. The cultures were grown at 30°C with shaking at 180 rpm. Samples of 1 ml were taken for RED and ACT assays. The samples were centrifuged for 10 min; both the supernatant and the pellet (the mycelium) were used for ACT assays, whereas only the mycelium was used for RED assays (40). The production of CDA on Oxoid nutrient agar medium was evaluated by a bioassay using *Micrococcus luteus* as the indicator (41).

Tests for resistance to H_2O_2. Each strain was grown in liquid tryptic soy broth (TSB) medium. The mycelium was harvested, and the same weight of mycelium from each strain was used. Mycelial pellets were disrupted by repeated passage through a needle (21 gauge). The optical density of each sample was determined, to allow all inocula used to be equal. The inocula were added to 3.5 ml of liquid soft nutrient agar (SNA) (37); this was poured onto R2YE plates for agar diffusion assays. A 4-mm-diameter cylinder was removed from the center of each plate, and 100 μl of 30% (8.8 M) H_2O_2 was poured into the hole. Results were read after 5 days of growth at 30°C. The quantitative assay involved spreading of dilutions of the disrupted mycelium onto R2YE medium supplemented or not with 8.8 mM H_2O_2. Colonies were counted after 5 days of growth.

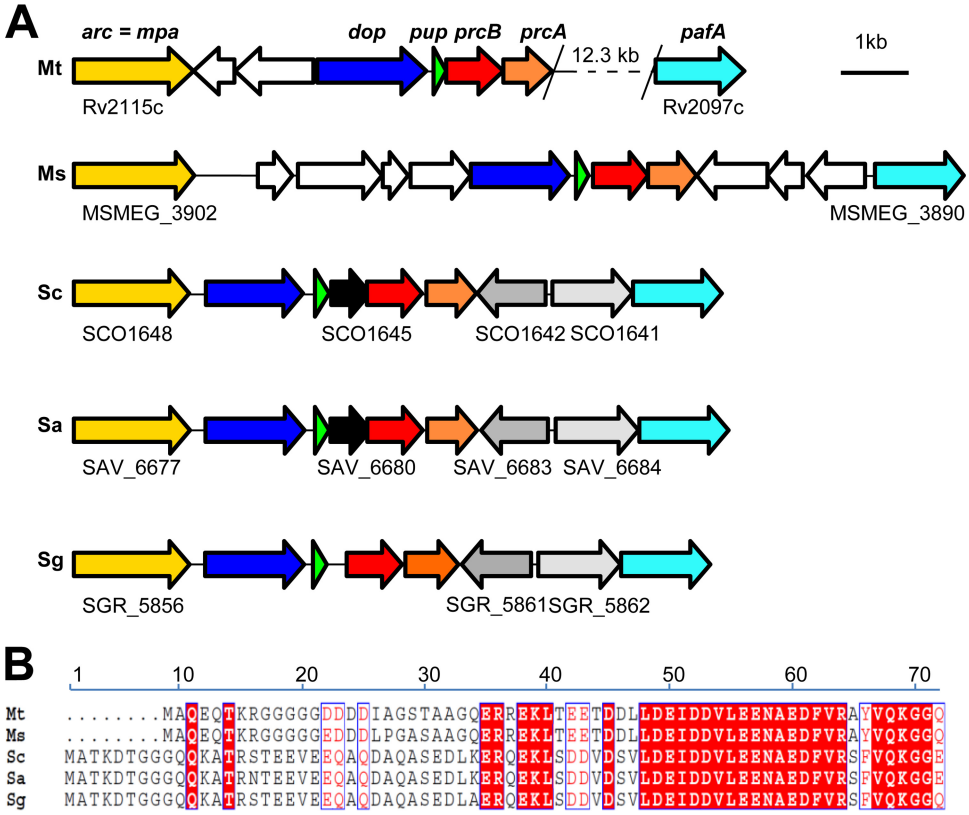

FIG 1 Pupylation and proteasome genes from mycobacteria and streptomycetes. (A) Comparison of the regions containing the pupylation and proteasome genes in *Mycobacterium tuberculosis* (Mt), *Mycobacterium smegmatis* (Ms), *Streptomyces coelicolor* (Sc), *Streptomyces avermitilis* (Sa), and *Streptomyces griseus* (Sg). Genes represented by arrows of the same color are orthologues, except those represented by white arrows, which are not syntenic in actinobacteria. The only difference between *Streptomyces* strains is the presence, in some strains, of a gene encoding a putative endonuclease VII (SCO1645 in *S. coelicolor* and SAV_6680 in *S. avermitilis*). The proteins SCO1641 (a putative transporter of the MFS family), SCO1642 (a putative LacI-like repressor), and SCO1645, and their orthologues in other *Streptomyces* species, are not predicted to be involved in functions related to pupylation or proteasomal degradation. (B) Alignment of Pup proteins from these five species.

RESULTS

Expression of pupylation and proteasome genes in *S. coelicolor*. The actinobacterial 20S proteasome consists of a proteolytic compartment assembled from the PrcA and PrcB proteins. Another protein, called Arc (for AAA^+ ATPase forming ring-shaped complexes) or Mpa (mycobacterial proteasomal ATPase) in mycobacteria, is also associated with this proteasome. Orthologues of the mycobacterial genes involved in pupylation (*pup*, *dop*, and *pafA*) are present in the genomes of *Streptomyces* spp., in which they are clustered with genes encoding components of the proteasome (*arc*, *prcA*, and *prcB*) (Fig. 1A). The organization of the locus is mostly conserved among streptomycetes. The products of the pupylation and proteasome genes in *S. coelicolor* are similar (70 to 92% amino acid similarity) to those in mycobacteria. However, there is a marked difference in that the Pup protein in *S. coelicolor* has a C-terminal glutamate residue, whereas the corresponding position in the mycobacterial protein is occupied by a glutamine residue (Fig. 1B). Dop is therefore not required for the deamidation of *S. coelicolor* Pup before its linkage to a target protein. The conservation of Dop in *S. coelicolor* might be explained by its possible role in depupylation, as shown in *Mycobacterium* (18).

We studied the transcription of the pupylation (*pup*, *dop*, and *pafA*) and proteasome (*prcA*, *prcB*, and *arc*) genes during *S. coelicolor* development by reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (see Fig. S1 in the supplemental material). Specific transcripts of all six genes were detected at each of the time points tested (24 h, 48 h, and 72 h). We also made use of some of the transcriptomic data sets available for *S. coelicolor* M145 in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) (42) (see the supplemental material). This analysis confirmed the transcription of the pupylation and proteasome genes at various stages of growth and development and showed that they were among the 5% of genes with the highest transcription levels. In *S. coelicolor*, the *pup*, *dop*, *prcB*, and *prcA* genes are in the same orientation, separated by only short intergenic regions or even overlapping (e.g., SCO1645-*prcB*), suggesting possible cotranscription. Indeed, the cotranscription of these genes was predicted by an *in silico* analysis (43). An analysis of the transcriptome sequencing (RNA-seq) data sets available from the GEO showed that many reads overlapped two consecutive genes in this region, suggesting that these genes are indeed cotranscribed (see the supplemental material). A semiquantitative analysis of protein abundance was performed by Thomas et al. for *S. coelicolor* M145 (44). The results obtained showed that Arc, Dop, PcrA, PcrB, and PafA were among 650 proteins detected in all time courses and that these proteins were relatively abundant.

Pupylation in *S. coelicolor*. We searched for Pup-tagged proteins in *S. coelicolor*. An N-terminally His_6-tagged version of Pup

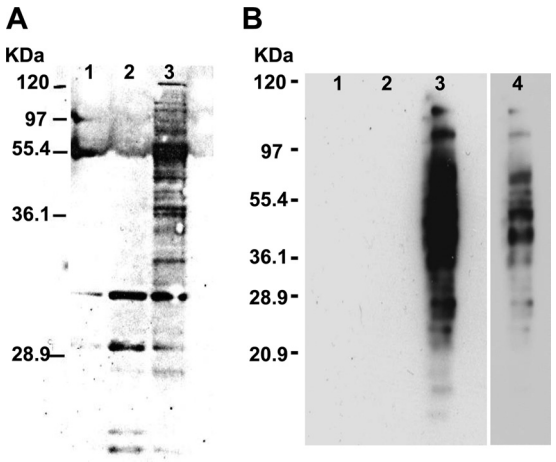

FIG 2 Immunoblots for the detection of pupylated proteins with anti-His_6 antibodies. (A) Proteins extracted from mycelium grown in liquid R2YE medium for the wild-type strain (lane 1), the wild-type strain harboring the empty vector pSET152 (lane 2), and the wild-type strain harboring pSET-E*-His-*pup* (lane 3). (B) Proteins extracted from mycelium grown in liquid YEME medium for the wild-type strain (lane 1), the wild-type strain harboring the empty vector pSET152 (lane 2), and the wild-type strain harboring pSET-E*-His-*pup* with the same exposure time as that for lanes 1 and 2 (lane 3) and a shorter exposure time (lane 4).

was produced in *S. coelicolor*, allowing subsequent detection of pupylated proteins with anti-His_6 antibodies. As *pup* was expressed at different stages of *S. coelicolor* growth and development, the gene encoding His_6-Pup was cloned under the control of the constitutive promoter *ermE** (45); the construct was inserted into the integrative vector pSET152, yielding pSET-E*-His-*pup*. This construct complemented the *pup* deletion mutant (see below). The wild-type *S. coelicolor* strain and strains harboring pSET152 or pSET-E*-His-*pup* were each grown in two different liquid media (YEME and R2YE) for 45 h. Protein samples were prepared from each of these cultures and analyzed by immunoblotting using an anti-His_6 antibody (Fig. 2). Many signals were observed with the extracts of the strain expressing His_6-Pup grown in both media. No or few detectable signals were observed under the same experimental conditions for samples from either the wild type or the strain harboring the empty vector. The few signals observed in these control samples may have been due to histidine-rich proteins. These experiments indicated that protein pupylation occurs in *S. coelicolor* and that an apparently large number of proteins are targeted by this system.

Identification of pupylated proteins in *S. coelicolor*. We undertook a proteome-wide analysis of pupylated proteins in *S. coelicolor* to check that Pup bound covalently to lysine residues and to identify some of the proteins tagged. Our analysis was based on previous studies in mycobacteria showing the binding of a GGE tripeptide tag to lysine residues in tryptic digests of pupylated proteins, associated with a +243-Da shift (26–28). The C-terminal sequence of the *S. coelicolor* Pup is KGGE, so we expected pupylation to lead to the same mass shift for trypsin-digested pupylated peptides. For this proteome-wide study, we used *S. coelicolor* strains (wild type and a *pup* deletion mutant) harboring the pSET-E*-His-*pup* vector. In these strains, His_6-tagged Pup was linked to a large number of proteins. These *S. coelicolor* strains grown in two different liquid media (YEME and R2YE) presented two different profiles on immunoblots with the anti-His_6 antibody (Fig. 2). We therefore studied the pupylomes for each of these two media. Ni-NTA affinity chromatography was used to enrich cell lysates in His-tagged pupylated proteins. The eluted fractions containing tagged proteins were then pooled for each culture and digested with trypsin. The resulting peptides were analyzed by LC-MS/MS. Proteins were identified by at least two unique peptides with probability scores of >95%.

We identified 467 proteins in the sample from the wild-type strain harboring pSET-E*-His-*pup* grown in YEME medium and 1,579 proteins in the sample from the *pup* deletion mutant strain harboring pSET-E*-His-*pup* grown in R2YE medium. A +243-Da modification, which could be attributed to the presence of the GGE tag on a lysine residue, was detected on 23 peptides, corresponding to 20 proteins (Table 1). Both Mascot and InSpecT proteomic search engines were used for these analyses. The pupylation tag was confirmed by a detailed inspection of the spectra of the peptides. The number of proteins with identifiable pupylation sites was small, possibly because Dop may be copurified with the pupylome, leading to the depupylation of some proteins, as previously reported (24).

Three pupylated proteins (GroEL2, AhpC, and SCO4496) were detected in both media. Six pupylated proteins were detected in the YEME pupylome, and 11 were detected in the R2YE pupylome. The 20 pupylation targets identified belong to 11 of the 23 Cluster of Orthologous Groups (COG) functional groups (Table 1). The functional group most frequently identified was posttranslational modification, protein turnover, and chaperone functions (O); four of the pupylation target proteins belonged to this group. Orthologues of seven of the 20 proteins (AcpP, FabF, PurA, Tuf1, GroEL1, AhpC, and SCO6042) were also identified in at least one of the pupylomes reported for *M. tuberculosis*, *M. smegmatis*, *R. erythropolis*, or *C. glutamicum* (26–30). However, 10 proteins pupylated in *S. coelicolor* were not described in any of the mycobacterial, *Rhodococcus*, and *Corynebacterium* pupylome analyses despite their conservation in these genera. SCO6042 and SCO3629 were the only proteins pupylated in *S. coelicolor* for which homologous proteins were also found to be pupylated in three of the four other actinobacterial species (Table 1; see also Fig. S2 in the supplemental material). For SCO6042, the same lysine was pupylated in *S. coelicolor* and *R. erythropolis*, but a different lysine was found to be pupylated in the two mycobacteria. For SCO3629, the lysine pupylated in *S. coelicolor* was found to be conserved in the three other actinobacteria, but a different lysine was pupylated in these other strains.

Morphological differentiation of pupylation- and proteasome-deficient mutants. Proteolysis is linked to the control of *Streptomyces* differentiation (35, 36, 46). The pupylation-directed degradation of proteins by the 20S proteasome may therefore be involved in differentiation. We investigated the role of pupylation in *S. coelicolor* by inactivating genes encoding Pup or components of the 20S proteasome. A *pup* mutant was constructed, in which most of the *pup* coding sequence was deleted. A proteasome-deficient mutant was constructed by disrupting *prcB* by the insertion of pOJ260, conferring apramycin resistance. This insertion abolished the transcription of *prcA* (data not shown) but should have no effect on the expression of genes other than *prcB* and *prcA*, as the gene downstream from *prcA* is in the opposite orientation (Fig. 1).

The wild-type strain and the *pup* and proteasome mutants

TABLE 1 Pupylated proteins identified in *S. coelicolor*

S. coelicolor gene locus	Protein name	Annotation	Detected pupylated peptide(s)[a]	Pupylated lysine	Medium[b]	Orthologue[c] in:				Search engine(s)[d]	COG category
						M. tuberculosis	*M. smegmatis*	*R. erythropolis*	*C. glutamicum*		
SCO1814	InhA/FabI	Putative enoyl-(acyl carrier protein) reductase	(R)DLGK(+243)QNIR(C)	K177	R	Rv1484	MSMEG_3151	RER_30750	cg2342	MI	I
SCO2090	FtsI	Cell division protein	(K)TGTANRVDPATGK(+243)(Y)	K582	R	Rv2163c	MSMEG_4233	RER_35580	cg2375	M	M
SCO2318		Putative glycosyl transferase	(R)TEHPRAHLVVVGK(+243)(R)	K293	R		MSMEG_6484		cg0481	M	M
SCO2389	AcpP	Acyl carrier protein	(M)AATQEEIVAGLAEIVNEIAGIPVEDVK(+43)LDK(+243)(S)	K31	Y	Rv2244	MSMEG_4326	RER_36730	cg2473	MI	I
SCO2390	FabF	3-Oxoacyl-(acyl carrier protein) synthase II	(R)K(+243)VLGDDVDHM(+16)AVSGTK(S)	K333	R	Rv2246	MSMEG_4328	RER_36740	cg3178	MI	I
SCO2950	HupA	Nucleoid-associated heat-unstable (HU) protein	(K)DADAVLAAFAEVVGDIVSK(+43)GDEK(+243)(V), (K)DADAVLAAFAEVVGDIVSK(+243)GDEK(V)	K38	Y	Rv2986c	MSMEG_2389			MI	L
			(K)DADAVLAAFAEVVGDIVSKGDEK(+243)(V)	K42							
SCO3373	ClpC	Clp family ATP-binding protease	(K)IAGVRRDK(+243)(E)	K431	R	Rv3596c	MSMEG_6091	RER_06460	cg3079	M	O
SCO3581		Conserved hypothetical protein	(K)IVNPK(+243)VAGQAEGGR(V)	K134	Y	Rv3688c	MSMEG_6214	RER_04620	cg0335	MI	S
SCO3629	PurA	Adenylosuccinate synthetase	(K)IGTTGRGIGPTYADK(+243)(I)	K139	R	Rv0357c	MSMEG_0759	RER_14280	cg3063	M	F
SCO3878	DnaN	DNA polymerase III, beta chain	(R)EFLWK(+243)PENPDISAVALVPAK(T)	K182	Y	Rv0002	MSMEG_0001	RER_00020	cg0004	MI	L
SCO4296	GroEL2	60-kDa chaperonin 2	(K)VTLGPKGRNVVLEK(+243)(K)	K41	R/Y	Rv3417c	MSMEG_1583	RER_19240	cg3011	M	O
SCO4496		Putative acetyltransferase	(R)FYESC(+57)GYK(+243)EVGRVPGAIR(V)	K147	R/Y					MI	K
SCO4662	Tuf1	Elongation factor Tu-1	(R)TVGAGQVTKINK(+243)(−)	K397	R	Rv0685	MSMEG_1401	RER_17640	cg0587	MI	J
SCO4762	GroEL1	60-kDa chaperonin	(K)DDTTIVDGAGK(+243)RDEVQGR(I)	K338	Y	Rv0440	MSMEG_0880	RER_15260	cg3011	MI	O
			(K)GQGFNAATGEYGDLVK(+243)AGVIDPVK(V)	K490							
SCO5032	AhpC	Alkyl hydroperoxide reductase	(K)GKEFQQINHK(+243)TYEGQWK(V)	K31	R/Y	Rv2428	MSMEG_4891	RER_27870	cg2736	MI	O
SCO5679		Putative aldehyde dehydrogenase	(R)EAVDAGAK(+243)LLTGGK(R)	K351	R		MSMEG_2163	RER_44340	cg0067	M	C
SCO5748	OsaA	Putative sensory histidine kinase	(K)GDLSKKITVDAK(+243)(G)	K813	R				cg0483	M	T
SCO6042		Conserved hypothetical protein	(R)AGYADEVQELFDQLAAK(+243)DVTIR(G)	K62	Y	Rv2676c	MSMEG_2782	RER_28490	cg2079	MI	S
SCO7343	HemC1	Porphobilinogen deaminase 1	(R)K(+243)LTDLPR(G)	K113	R	Rv0510	MSMEG_0953	RER_16420	cg0498	MI	H
SCO7442		Putative uncharacterized protein	(K)AVSMSDPNKDTNK(+243)(K)	K22	R					M	S

[a] Amino acid modifications are indicated as follows: +16, oxidation; +57, carbamidomethyl; +43, carbamyl; +243, pupylation.

[b] Y, YEME medium; R, R2YE medium.

[c] Orthologous proteins detected as confirmed pupylated targets in mycobacterial, *Rhodococcus*, or *Corynebacterium* pupylomes (10, 11, 19, 25, 50) are underlined. Orthologous proteins identified in these organisms were obtained by a BLAST search through the MAGE genome browser (https://www.genoscope.cns.fr/agc/microscope/home/index.php).

[d] The search engine used was Mascot (M), InSpecT (I), or both (MI).

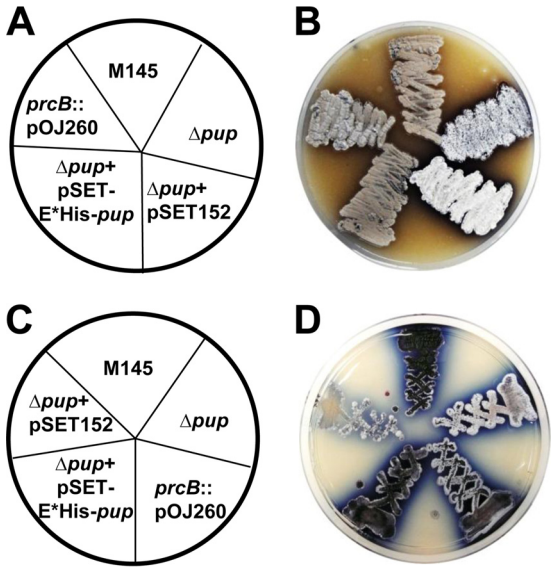

FIG 3 Morphological differentiation on solid SFM (A and B) and R2YE (C and D) media. The wild-type strain (M145), the *pup* mutant (Δ*pup*), the *pup* mutant with the empty vector pSET152 (Δ*pup*+pSET152), the *pup* mutant with pSET-E*-His-*pup* (Δ*pup*+pSET-E*His-*pup*), and the proteasome mutant (*prcB*::pOJ260) are shown after 5 days of growth.

were grown on solid soy flour-mannitol (SFM) medium (37) and the colonies obtained were compared in terms of their appearance. After 5 days of growth, the mycelium of the *pup* deletion mutant was much less pigmented than that of the wild-type strain (Fig. 3). The medium surrounding the *pup* deletion strain was darker than that surrounding the wild-type strain, suggesting a higher level of production of pigmented secondary metabolites. The wild-type phenotype was restored in the mutant strain by transformation with pSET-E*-His-*pup*. No such complementation was observed with the empty vector (pSET152). We can therefore conclude that complementation was due to *pup* expression. The growth and differentiation of the proteasome-deficient mutant on SFM agar did not appear to be different from those of the wild type (Fig. 3B). We monitored the appearance of the mycelium of each strain over a period of 10 days. The phenotype of the *pup* mutant remained unchanged throughout this longer incubation (see Fig. S3 in the supplemental material). The differences observed were therefore due to a defect in differentiation and not to a delay in the differentiation of the *pup* mutant.

A second medium, R2YE, a rich medium also used to visualize the effects of mutation on *Streptomyces* differentiation (34, 47), was also used to study the effects of pupylation and proteasome defects. Again, the mycelia of the five strains grown on solid R2YE medium were examined by eye (Fig. 3D). The *pup* deletion mutant was clearly different from the wild-type strain, with a much less pigmented aerial mycelium and a lower level of production of pigmented secondary metabolites. The proteasome-deficient mutant appeared to be almost unaffected, displaying only a slightly lower level of mycelium pigmentation than the wild-type strain. In the *pup* deletion mutant, a phenotype similar to that of the wild type was restored by the expression of His-tagged Pup. This restoration could clearly be attributed to Pup expression, as it was not observed for the *pup* deletion mutant containing the empty vector.

Differences in aerial mycelium pigmentation may be linked to sporulation defects (48–50). Consistent with this hypothesis, we found that the yield of viable spores from the *pup* deletion strain in confluent cultures on R2YE or SFM plates was ~1% that of the wild type (data not shown), suggesting that spore formation was impaired.

The differences in colony morphology upon visual examination and in viable spore counts led us to use scanning electron microscopy (SEM) to evaluate aerial mycelia and spore formation in these strains. After 5 days of growth on solid R2YE medium, we harvested samples from each of the five strains and prepared them for SEM (Fig. 4). The wild-type strain displayed three dominant features: (i) the aerial mycelium was abundant, (ii) many spore chains were visible, and (iii) the different stages of spore development were represented as visible immature and mature spore chains. The *pup* deletion mutant also had an abundant aerial mycelium, but many of the hyphae were collapsed and thin, and few of the typical spore chains were observed. In the *pup* deletion mutant transformed with the empty pSET152 vector, spore chains were even scarcer. Complementation with the *pup* gene under the control of the constitutive *ermE** promoter (pSET-E*-His-*pup*) restored a phenotype similar to that of the wild-type strain. The proteasome-deficient mutant displayed impaired aerial mycelium development on R2YE medium (Fig. 4).

On SFM medium, the *pup* deletion mutant also displayed phenotypic impairment, with far fewer coils and spore chains than the wild type (see Fig. S4 in the supplemental material). The expression of His-Pup restored normal sporulation. In contrast to the results obtained with R2YE medium, the proteasome-deficient mutant presented normal aerial mycelium development and sporulation, highlighting the existence of a medium-dependent phenotype in this mutant (see Fig. S4 in the supplemental material).

Production of secondary metabolites by pupylation- and proteasome-deficient mutants. The production of pigmented secondary metabolites by the *pup*-deficient mutant on R2YE medium differed from that of the wild-type strain. We therefore studied the involvement of the pupylation and proteasome systems in antibiotic production. Under typical laboratory conditions, *S. coelicolor* produces two pigmented antibiotics, RED and ACT, together with the nonpigmented CDA (37). We tested the effect of the absence of pupylation or of the proteasome on the production of these antibiotics. For CDA, we observed no significant difference in the diameters of the inhibition zones surrounding the colonies of the five strains challenged with *Micrococcus luteus* on a plate (see Fig. S5 in the supplemental material).

ACT and RED production levels were monitored over 7 days of growth in liquid R2YE medium (Fig. 5). The levels of both ACT and RED produced by the *pup* mutant (with or without the empty vector) were substantially lower than those of the wild-type strain over the entire 7-day period. The production of His-Pup in the *pup* mutant restored ACT production to levels similar to those in the wild-type strain. For RED production, the restoration was only partial. For the proteasome mutant, only ACT production differed from that of the wild-type strain, and the difference was modest (the mutant produced half the amount produced by the wild-type strain).

Sensitivity of pupylation- and proteasome-deficient mutants to oxidative stress. Oxidative stress has been linked to pupylation and the proteasome in both *Mycobacterium* (9, 10, 18, 51, 52) and *Streptomyces* (12). We therefore also assessed the sensitivity of

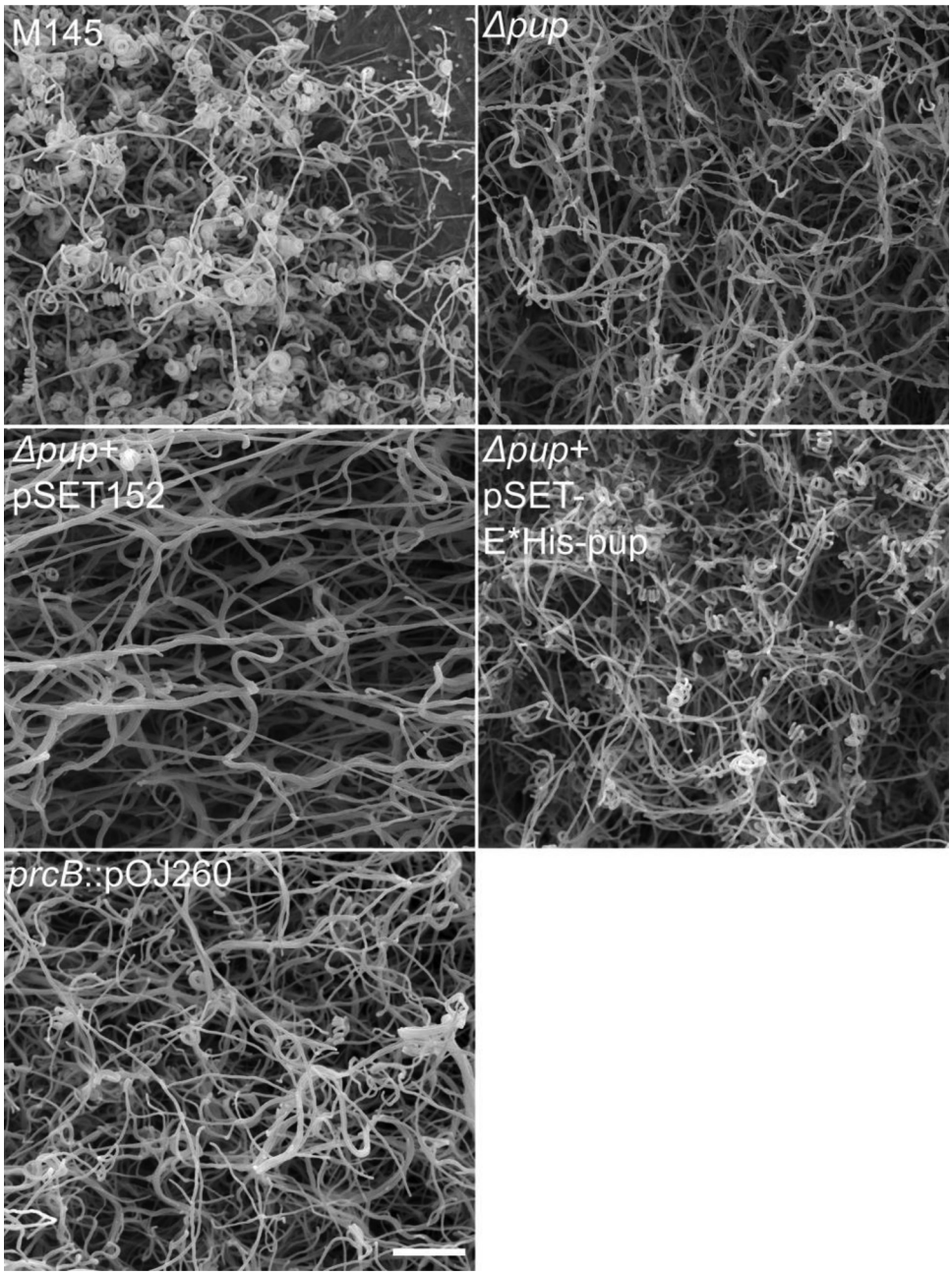

FIG 4 Scanning electron microscopy of mycelium grown on R2YE medium. Scanning electron micrographs of surface cultures grown on R2YE medium for 5 days are shown for the wild-type strain (M145), the *pup* mutant (Δ*pup*), the *pup* mutant with the empty vector pSET152 (Δ*pup*+pSET152), the *pup* mutant with pSET-E*-His-*pup* (Δ*pup*+pSET-E*His-*pup*), and the proteasome mutant (*prcB*::pOJ260). The same magnification is used for all images. Bar, 10 μm.

each of the five *S. coelicolor* strains to oxidative stress. Hydrogen peroxide was used as an oxidant in solid R2YE and SFM media. We first observed the inhibition of growth for each strain following exposure to a gradient of concentrations of hydrogen peroxide in an agar diffusion test. The *pup* deletion mutants presented a halo of inhibition larger than those of the other strains on R2YE medium (Fig. 6A) and on SFM medium (data not shown). We then evaluated the effects of oxidative stress by plating disrupted mycelia from the five strains onto R2YE solid medium with and without hydrogen peroxide. Colonies were counted after 5 days of growth (Fig. 6B). The *pup* deletion mutant (with or without the empty vector) was much more sensitive to hydrogen peroxide than the other strains. This sensitivity of the *pup* mutant to hydrogen peroxide was complemented by introducing a copy of the His-*pup* gene on pSET152. The sensitivity of the proteasome mutant to H_2O_2 was similar to that of the wild-type strain (Fig. 6B).

DISCUSSION

Our objectives were to determine whether pupylation occurs in *Streptomyces* and, if so, to investigate its role in morphological and physiological differentiation, comparing the results obtained with those for the proteasome. We chose to perform the study with *S.*

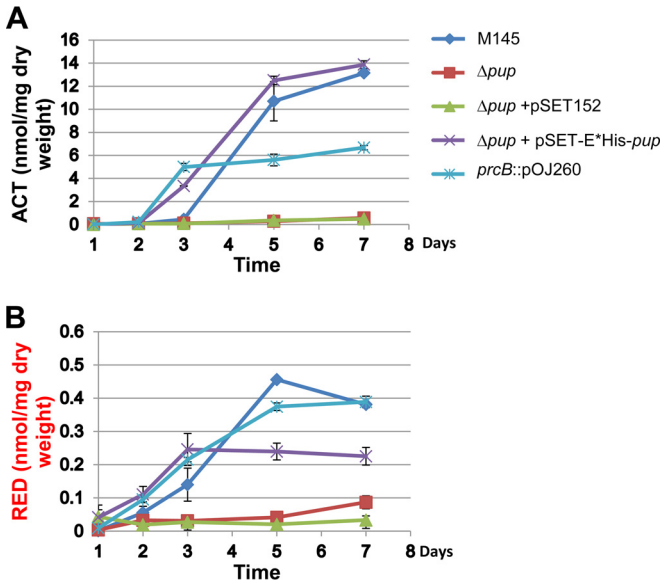

FIG 5 Time courses of actinorhodin (ACT) and prodiginine (RED) production in liquid R2YE medium. The production of ACT and RED is shown for the wild-type strain (M145), the *pup* mutant (Δ*pup*), the *pup* mutant with the empty vector pSET152 (Δ*pup* + pSET152), the *pup* mutant with pSET-E*-His-*pup* (Δ*pup* + pSET-E*His-*pup*), and the proteasome mutant (*prcB*::pOJ260).

coelicolor, the model organism used for most studies of development in filamentous actinobacteria. In streptomycetes, as in mycobacteria, the genes involved in pupylation and those encoding components of the proteasome are clustered together on the chromosome. In *S. coelicolor*, these genes were transcribed at various stages of development on solid medium and at all time points tested in liquid culture. This finding is consistent with data from previous proteome studies in *S. coelicolor*, which have shown that all the proteins of the proteasome and the pupylation process are present at various times during growth, including the early stages (44).

We then demonstrated that the pupylation process was active in *S. coelicolor* and that numerous proteins were modified by this process. We identified 20 pupylation targets in *S. coelicolor*. Seven of these target proteins have homologues that are also pupylated in *Mycobacterium*, *R. erythropolis*, or *C. glutamicum* (26–30). These homologous proteins were, in some cases, modified at different positions in different actinobacteria (see Fig. S2 in the supplemental material). As a result, the currently available prediction programs based on the mycobacterial pupylome (53–55) do not predict pupylation sites in *S. coelicolor* proteins accurately. Indeed, the three available prediction programs predicted only 6 to 11 of the 22 modified lysine residues identified in *S. coelicolor* pupylated proteins (see Table S3 in the supplemental material). One of the 20 pupylated proteins identified in this study, AhpC (SCO0465), has already been shown to accumulate in a *S. coelicolor* proteasome-deficient mutant and in *arc*, *dop*, and *pup* insertion mutants (12). These observations are consistent with the degradation of pupylated proteins by the proteasome.

Investigations of the consequences of *pup* deletion for morphological differentiation showed that the *pup* mutant displayed a major impairment of spore formation on R2YE and SFM media. On the contrary, for the proteasome mutant, differentiation was not affected on SFM medium, but spore formation was reduced on R2YE medium. The *pup* mutant produced significantly less of the secondary metabolites ACT and RED than the wild type in liquid R2YE medium. For the proteasome-deficient mutant, the production of ACT and RED was not affected (e.g., production of ACT in liquid R2YE medium) or was only slightly affected (e.g., halving of the production of RED in liquid R2YE medium). Mao and coworkers also observed that a proteasome-deficient mutant grown on solid R2YE medium produced smaller amounts of pigmented secondary metabolites than the wild-type strain (13).

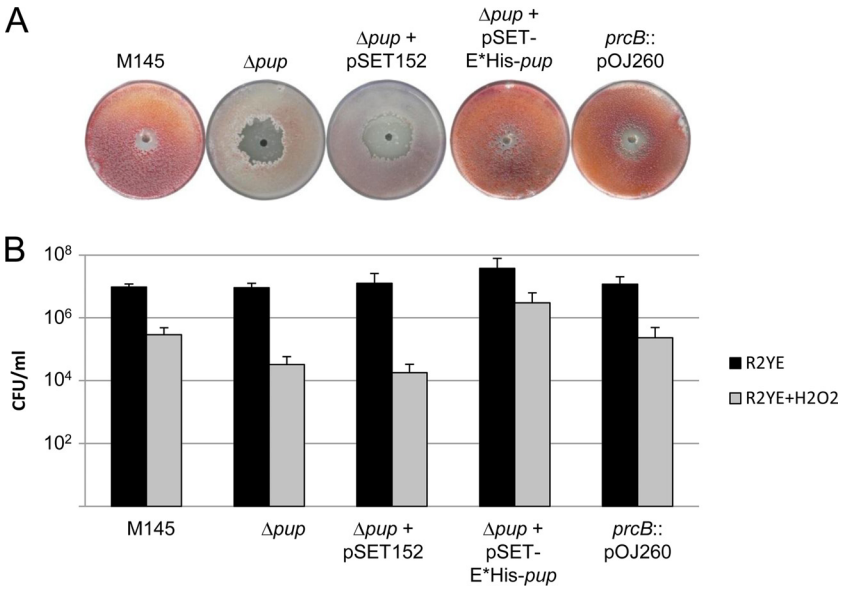

FIG 6 Response to oxidative stress. The wild-type strain (M145), the *pup* mutant (Δ*pup*), the *pup* mutant with the empty vector pSET152 (Δ*pup* + pSET152), the *pup* mutant with pSET-E*-His-*pup* (Δ*pup* + pSET-E*His-*pup*), and the proteasome mutant (*prcB*::pOJ260) were exposed to oxidative stress caused by H_2O_2 on R2YE medium. (A) Diffusion assay showing the inhibition zone caused by H_2O_2. (B) CFU counts on medium with or without H_2O_2.

Consistent with our results, the preliminary data of Liu and coworkers (56) led them to conclude that pupylation was involved in the regulation of antibiotic production in *S. coelicolor*. Some of the proteins that we identified as pupylation targets in *S. coelicolor* play a role in regulating morphological differentiation or antibiotic production. This is the case, for instance, for OsaA (57) and FtsI (58), but further studies are required to link the phenotype of the pupylation-deficient mutant for morphological and metabolic differentiation with the fate of particular target proteins.

Previous studies have established a link between oxidative stress, pupylation, and proteasomes in both *Mycobacterium* (9, 10, 18, 51, 52) and *Streptomyces* (12). We therefore compared the resistance of the pupylation- and proteasome-deficient mutants to oxidative stress with that of the wild-type strain. We found that the *pup* deletion mutant was much more sensitive than the wild type to hydrogen peroxide on R2YE and SFM media. Under the same conditions, the sensitivity of the proteasome-deficient mutant to hydrogen peroxide was similar to that of the wild type. In a previous study (12), De Mot and coworkers studied the sensitivities of a *S. coelicolor* proteasome-deficient mutant and *arc*, *dop*, and *pup* knockout mutants to oxidative agents. They used an agar diffusion test on TSB medium. Under these conditions, those researchers observed no difference in sensitivity to hydrogen peroxide (at concentrations of up to 0.5 M). There are several possible reasons for the differences between our observations for the *pup* mutant and those of the previous study. For example, the medium used was not the same, and the concentration of hydrogen peroxide used in the agar diffusion test was much higher in our case (8.8 M versus 0.5 M). Our results were confirmed by a CFU assay assessing sensitivity to hydrogen peroxide.

De Mot and coworkers observed greater resistance to cumene hydroperoxide on TSB medium in an agar diffusion system for a *S. coelicolor* proteasome-deficient mutant and for *arc*, *dop*, and *pup* knockout mutants (12). In *M. tuberculosis*, the pupylation and proteasome mutants were found to be more resistant to hydrogen peroxide than the wild-type strain but more sensitive to other forms of oxidative stress (9, 10). The different patterns of behavior observed for the mutants of different bacteria exposed to hydrogen peroxide or cumene hydroperoxide suggest that different defense systems are active against specific classes of exogenous peroxides, as previously observed (12, 59).

Finally, the phenotype of the *S. coelicolor pup* deletion mutant reveals the involvement of pupylation in the regulation of *Streptomyces* differentiation and resistance to oxidative stress. Our results are consistent with those of Compton et al., who found that a *S. coelicolor pafA*-null strain displayed defects of both sporulation and secondary metabolism and who also presented evidence linking pupylation and the oxidative stress response in *S. coelicolor* (60). The differences in the phenotypes of the *pup* and proteasome mutants observed in our study indicate that pupylation may have other roles in addition to its known proteasome-associated function. This conclusion is consistent with previous observations (26, 29) that not all pupylated proteins are degraded. Furthermore, pupylation occurs in corynebacteria, which do not have a proteasome (30). Thus, in addition to targeting proteins to the proteasome for turnover, pupylation, like ubiquitination, is probably also involved in other functions.

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